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RESEARCH

Protective effect of *Bifidobacterium animalis* CGMCC25262 on HaCaT keratinocytes

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Abstract

Bifidobacteria are the most prevalent members of the intestinal microbiota in mammals and other animals, and they play a significant role in promoting gut health through their probiotic effects. Recently, the potential applications of *Bifidobacteria* have been extended to skin health. However, the beneficial mechanism of *Bifidobacteria* on the skin barrier remains unclear. In this study, keratinocyte HaCaT cells were used as models to evaluate the protective effects of the cell-free supernatant (CFS), heat-inactivated bacteria, and bacterial lysate of *Bifidobacterium animalis* CGMCC25262 on the skin barrier and inflammatory cytokines. The results showed that all the tested samples were able to upregulate the transcription levels of biomarker genes associated with the skin barrier, such as hyaluronic acid synthetase (HAS) and aquaporins (AQPs). Notably, the transcription of the hyaluronic acid synthetase gene-2 (HAS-2) is upregulated by 3~4 times, and AQP3 increased by 2.5 times when the keratinocyte HaCaT cells were co-incubated with 0.8 to 1% CFS. In particular, the expression level of Filaggrin (FLG) in HaCaT cells increased by 1.7 to 2.7 times when incubated with Bifidobacterial samples, reaching its peak at a concentration of 0.8% CFS. Moreover, *B. animalis* CGMCC25262 also decreased the expression of the proinflammatory cytokine RANTES to one-tenth compared to the levels observed in HaCaT cells induced with tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ). These results demonstrate the potential of *B. animalis* CGMCC25262 in protecting the skin barrier and reducing inflammatory response.

Keywords *Bifidobacterium animalis* · Skin barrier · Probiotic role · Keratinocyte HaCaT · Cosmetic material

Introduction

Lactic acid bacteria, which are commonly used as starter cultures in dairy fermentation processes, play a crucial role in enhancing the sensory qualities, texture, and shelf life of fermented products (Zapaśnik et al. 2022). In addition to their long history of safe use in the food industry, lactic acid bacteria, particularly certain members of the intestinal commensals *Lactobacillus*, have been shown to have beneficial effects on the health of host. These effects include reducing serum cholesterol levels (De Rodas et al. 1996), balancing intestinal microflora (Lidbeck and Nord 1991), and exerting immunomodulatory effects (Kalliomaki et al. 2003). *Bifidobacterium* is a member of the actinomycetes genus. As one of the first microbes to colonize the human gastrointestinal tract, *Bifidobacterium* is thought to have beneficial health effects on the host (Fijan 2014; O'Callaghan and van Sinderen 2016). Due to their health-promoting effects and the functionality of their metabolites, members of the *Lactobacillus* and *Bifidobacterium* genera are included as the functional ingredients in commercial probiotic products for human consumption (Wei et al. 2007; Gao et al. 2020; Zhang et al. 2023). According to the US Food and Drug Administration (FDA), probiotics are defined as "live microorganisms that, when administered in sufficient quantities, provide health benefits to the host." Normally, probiotics are administered orally, allowing the probiotic bacteria to come into direct contact with the intestinal epithelium and promote immune activity (Kemgang et al. 2014; Ren et al. 2015). Based on the definition and functionality of probiotics, there is potential for probiotics to be applied in the field of enhancing skin health.

The skin, which is the largest organ of the body, serves as the primary defense against infections. It establishes a

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crucial barrier between the organism and the external environment. The physical, chemical, microbial, and immune barrier components of the skin form an interactive system that contributes to cutaneous defense and the maintenance of skin microecological homeostasis (Magdalena et al. 2016; Lee and Kim 2022). However, the skin barrier is susceptible to damage in daily life due to external environmental factors such as temperature, pollution, and ultraviolet radiation. This damage results in the disorganization and loosening of keratinocytes, reduced sebum and water, and increased transepidermal water loss (TEWL) (Boada et al. 2010; Cushing and Phillips 2013; Gabriela et al. 2014; Kenji et al. 2020). Consequently, it can lead to skin issues such as wrinkles, dryness, glycosylation of the epidermis, and even skin diseases (Andrew and Richard 2011; Dainichi et al. 2014). Skin health is associated with the maintenance of tight junctions in the gut, which is achieved through communication via the skin-gut axis. This means that the modulating the microbiome with probiotics may provide benefits for skin inflammation and homeostasis (Arck et al. 2010). Clinical studies have already reported that probiotics may have positive effects on skin health, extending beyond gut well-being. These effects include improvements in conditions such as atopic eczema, atopic dermatitis, burn and scar healing, skin rejuvenation, and enhancement of the skin's innate immunity. These benefits stem from balancing the skin microbiome, enhancing the skin barrier, and overall improving the skin's appearance (Kemgang et al. 2014; Ren et al. 2015; Vasiliki and Mihalis 2019; Mohtashami et al. 2020; PueblaBarragan and Reid 2021; Tsai et al. 2021; Karampoor et al. 2022; Moysidis et al. 2022; Yu et al. 2022). Several *Lactobacillus* species have been reported to have probiotic effects, including antioxidant, moisturizing, and whitening capabilities in cosmetic products (Amaretti et al. 2013; Goderska 2019; Vasiliki and Mihalis 2019; PueblaBarragan and Reid 2021; Tsai et al. 2021; Zhao et al. 2021; Yu et al. 2022). The probiotics market is projected to grow at a rate of 12% in the next decade, primarily driven by North America (PueblaBarragan and Reid 2021). To address safety concerns related to the use of live microorganisms, skin care products now incorporate components derived from probiotic strains, including bacterial lysates, ferments, and filtrates. These substances are sometimes referred to as postbiotics and have been shown to have beneficial effects on the skin (Duarte et al. 2022; Gueniche et al. 2022; Vale et al. 2023). The well-known "*Bifida* ferment lysate," an ultrasound-inactivated suspension of *Bifidobacterium longum reuter*, has been shown to improve barrier function and decrease skin sensitivity (Gueniche et al. 2010). Additionally, studies have shown that cell lysates from *Lactobacillus bulgaricus*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, and *Bifidobacterium longum* can promote the healing of damaged skin (Mohtashami et al. 2020; Karampoor et al. 2022; Moysidis et al. 2022). Despite these findings, there is still a significant knowledge gap regarding the mechanisms through which probiotics and their ingredients impact skin health.

In this study, *Bifidobacterium animalis* CGMCC25262, which was originally isolated from adult feces, exhibited strong antioxidant activity. To investigate the potential application of *B. animalis* CGMCC25262 in skincare products, we utilized keratinocyte HaCaT cells as a model to evaluate its effects on the transcription and expression levels of biomarker genes associated with skin barrier function, as well as the pro-inflammatory cytokines in HaCaT cells. This was accomplished by co-incubating the cells with heat-inactivated bacterial cells, bacterial lysates, and CFS derived from the strain *B. animalis* CGMCC25262. The objective of this study was to establish the theoretical foundation for the development of a potentially innovative and environmentally friendly cosmetic ingredient for skincare products.

Materials and methods

Strains, culture conditions, and sample preparation

B. animalis W3 was isolated from the feces of healthy adults and stored in the China General Microbiological Culture Collection Center under the strain number *B. animalis* CGMCC25262.

B. animalis CGMCC25262 was routinely cultured in de Man, Rogosa and Sharpe (MRS) broth with 0.05% cysteine, statically at 37°C for 36 h. The concentration of Bifidobacterium was approximately 10^9 CFU/mL at the end of fermentation. CFS was collected by centrifugation at 6000 rpm for 5 min and filtered through a 0.22-μm filter. The CFS was serially diluted with High-Glu DMEM agent (Sparkjade, Jinan, CHN), and the resulting dilutions were labeled as sample 1. The bacterial cells were washed three times with phosphate-buffered saline (PBS) and then resuspended in High-Glu DMEM solution at s final concentration of approximately 10^9 CFU/mL. The bacterial cell suspensions were either boiled for 10 min or subjected to ultrasonic disruption (450 W, 5-s pulses on ice with 5-s intervals). After disruption, the suspensions were centrifuged at 10,000 rpm for 10 min to remove the cellular debris, resulting in the production of heat-inactivated bacteria (referred to as "sample 2") or bacterial lysate ("sample 3"). In addition, the bacterial cell lysate was also inactivated by boiling for 10 min, and this was designated as sample 4.

The human keratinocyte line HaCaT was cultured in High-Glu DMEM complete medium containing 10% (vol/vol) fetal bovine serum (Gibco, USA) and 1% (vol/vol) penicillin/streptomycin solution (100 U/mL of penicillin and 100 μg/mL of streptomycin, SparkJade, Jinan, CHN) at 37°C in a 5% CO_2 atmosphere.

Evaluation of keratinocyte HaCaT cell survivability

Cell viability was assessed using the CCK-8 assay, following the instructions (US Everbright® Inc, Suzhou, CHN). Keratinocyte HaCaT cells were detached using 0.25% trypsin-EDTA solution (SparkJade, Jinan, CHN) and then centrifuged at 1000 rpm for 5 min. The cells were collected and suspended in High-Glu DMEM complete medium, and then seeded in a 96-well plate at the concentration of 1×10^4 cells per well. They were then incubated for 24 h at 37°C. After washing with phosphate buffer saline (PBS, pH 7.2), the HaCaT cells were co-incubated with 100 µL of each sample prepared above (sample 1, 2, 3, 4, respectively) and 100 µL of High-Glu DMEM medium for 24 h. The cells were washed three times with PBS buffer, and 100 µL of 10% CCK-8 (diluted with High-Glu DMEM) was added and incubated for 1 h. The optical density (OD) at 450 nm was measured to assess the cell viability. The cells incubated with High-Glu DMEM medium alone were used as the control. Six parallels were established for each treatment. Data were expressed as mean ± standard deviation (SD).

HaCaT cells treated with the four samples were obtained as described above. The cells were washed three times with PBS buffer, and 100 µL of 0.35 mM H2O2 (diluted with High-Glu DMEM) was added and incubated for 0.5 h. Then cells were washed three times with PBS buffer, and 100 µL of 10% CCK-8 (diluted with High-Glu DMEM) was added and incubated for 1 h. The optical density (OD) at 450 nm was measured to assess the cell viability. The cells incubated with High-Glu DMEM medium alone were used as the control. Six parallels were established for each treatment. Data were expressed as mean ± standard deviation (SD).

Analysis of the transcription levels of the genes related to epidermal barrier in HaCaT cells

Keratinocyte HaCaT cells were seeded in six-well plates at a density of 1×10^6 cells/mL and cultured at 37°C for 24 h. After washing with PBS, the HaCaT cells were co-incubated with 2 mL of each sample or 2 mL of High-Glu DMEM medium, and then incubated for 24 h. The HaCaT cells were harvested, and total RNA was extracted from them using the SPAPKeasy Cell RNA Kit (SparkJade, Jinan, CHN), and cDNA was synthesized using the PrimeScript RT reagent kit (Takara, Dalian, CHN) according to the instructions. The gene transcription levels of hyaluronic acid synthetase (HAS), Human β-defensin (HBD), involucrin (IVL), Filaggrin (FLG), aquaporin 3 (AQP3), Glyceraldehyde-3-phosphate dehydrogenase (GADPH), macrophage derived chemokine (MDC), thymic and activating regulatory chemokine (TARC), Regulation of Activation Expression and Secretion by Normal T Cells (RANTES), interleukin 33 (IL-33), interleukin 25 (IL-25), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-α) were analyzed by real-time fluorescence quantitative PCR using 2× Universal SYBR Green Fast qPCR Mix. PCR amplification was conducted using the following protocol: initial denaturation at 95°C for 5 min followed by 40 cycles at 95°C for 15 s, 55°C for 15 s, and 72°C for 20 s. GAPDH was utilized as a reference gene, and the relative gene transcription was calculated using the $2^{-\Delta\Delta CT}$ formula. The untreated HaCaT cells were used as controls. Primers are listed in Table 1.

Attenuating effects of *B. animalis* samples on skin inflammatory cytokines in induced HaCaT model

HaCaT cells treated with the four samples were obtained as described in Section 2.3. After being washed three times with PBS, 2 mL of High-Glu DMEM containing a final concentration of 20 ng/mL TNF-α and IFN-γ was added to each respective well. The cells were then incubated for 2 h to induce damaged HaCaT cell models. HaCaT cells treated with only TNF-α and IFN-γ were used as the control group. HaCaT cells incubated solely in High-Glu DMEM were considered the normal group. Subsequently, total RNA or protein was extracted for the analysis of gene expression levels related to inflammatory cytokines.

Protein content and MAPKs pathway detection

The expression levels of FLG and P-ERK/ERK in HaCaT cells were assessed using Western blot analysis. HaCaT cells were treated with each sample, as described in Section 2.3. Total proteins were extracted according to the manufacturer's instructions (Beyotime, Shanghai, CHN). The protein extract was centrifuged at 12,000 ×g for 10 min at 4°C, and the resulting supernatant was collected.

Equal amounts of protein were separated by electrophoresis on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Then, the proteins were transferred to a polyvinylidene difluoride membrane (PVDF membrane, Millipore, Bedford, MA, USA) at 200 mA in transfer buffer (192 mM glycine and 25 mM Tris) for 2 h. The membrane was blocked with tris buffered saline Tween (TBST) buffer containing 5% skim milk for 1 h. The PVDF membrane was then incubated with primary antibodies (1:1000 dilution, ABclonal, CHN, Affinity Biosciences, CHN) overnight at 4°C, followed by horseradish peroxidase-conjugated secondary antibodies (1:10000 dilution, ABclonal, CHN). The Amersham Imager 680 (GE, USA) was used to capture images and analyze the gray values of FLG, Occludins (OCLN), Claudin 1 (CLDN 1), and P-ERK/ERK proteins, normalized to GAPDH.

Table 1 RT-qPCR primer

Gene	Forward (5'-3')	Reverse (from 5'-3')
GAPDH	CTTTGTCAAGCTCATTTCCTGG	TCTTCCTCTTGTGCTCTTGC
HAS-1	GTCTCCAGGGAGGGTATTTATTG	TCCTGATCACACAGTAGAAATGG
HAS-2	CTGGGACGAAGTGTGGATTATG	CTCCAACCATGGGATCTTCTTC
HAS-3	AGGTATGGCAGTAGAGGATGA	CCTGCTCACCATTGGAGAAT
IVL	GGCCACCCAAACATAAATAACC	CCTAGCGGACCCGAAATAAG
FLG	TGAAGCCTATGACACCACTGA	TCCCCTACGCTTTCTTGTCCT
AQP3	ACCAGCTTTTTGTTTCGGGC	ATGGAGGTGCCAATGACCAG
HBD-1	TCTGCTGTTTACTCTCTGCTTAC	GGCAGGCAGAATAGAGACATT
HBD-2	GATGCCTCTTCCAGGTGTTT	CCACAGGTGCCAATTTGTTTAT
HBD-3	CCAGGTCATGGAGGAATCATAAA	CGATCTGTTCCTCCTTTGGAAG
MDC	GTTGTCCTCGTCCTCCTTGC	GGAGTCTGAGGTCCAGTAGAAGTG
TARC	TGTTCGGACCCCAACAACAA	TAGTCCCGGGAGACAGTCAG
IL-33	TCAGGTGACGGTGTTGATGG	GGAGCTCCACAGAGTGTTCC
IL-25	CACACAAGCTAAGGAAACA	CTCTACCACAACCAGACT
TNF-α	CGCTCCCAAGAAGACAG	AGAGGCTGAGGAACAAGCAC
IL-6	CCGGGAACGAAAGAGAAGCT	AGGCGCTTGTGGAGAAGGA
RANTES	CCGCGGCAGCCCTCGCTGTCATCC	CATCTCCAAAGAGTTGATGTACTCC

Statistical analysis

The experiments were performed in triplicate. Statistical analysis was performed using unpaired two-tailed Student's *t*-tests, and the results are expressed as the mean ± standard deviation. Statistical significance between the normal/control and experimental groups was analyzed using GraphPad Prism 5. A *P*-value of less than 0.05 was considered statistically significant. $P<0.01$ was considered highly significant.

Results

Cell viability

Bifidobacteria naturally inhabit the human intestinal tract, where they produce various organic acids, such as lactic acid and acetic acid, through carbohydrate fermentation. This fermentation process results in a decrease in pH. To eliminate the potential harm caused by these organic acids, the CFS of *B. animalis* CGMCC25262 cultures was serially diluted with High-Glu DMEM. The various dilutions were then co-incubated with HaCaT cells for 24 h. The viability of HaCaT cells was assessed, as shown in Fig. 1.

The viability of HaCaT cells increased in conjunction with the concentration of CFS, within the range of 0.2~1%. The highest viability was achieved at 0.8%, resulting in an 11.2% increase compared to untreated HaCaT cells. This indicates that the CFS has the ability to promote the growth of HaCaT cells, with statistically significant effects observed at CFS concentrations of 0.2% and 0.8% ($P<0.001$). Concentrations of CFS exceeding 3% had a detrimental impact on HaCaT cells, resulting in decreased cell viability.

Similarly, the survival of HaCaT cells co-incubated with heat-inactivated bacteria, bacterial lysate, and heat-inactivated bacterial lysate was also higher than that of HaCaT cells alone. Notably, the bacteria have particularly strong promoting effects on the proliferation of HaCaT cells, suggesting that bacterial cell wall fragments, heat-inactivated bacterial cells, and their active metabolites have beneficial effects on keratinocyte HaCaT cells (Fig. 1). At the same time, all the samples can alleviate the oxidative damage caused by hydrogen peroxide (Fig. 1).

Upregulatory effects of Bifidobacterial samples on genes related to skin physical barrier and FLG protein

The skin is the first line of defense against the external environment and plays a role in barrier function. A number of specific genes associated with skin physical barrier of the skin were identified as being susceptible to environmental changes. To assess the protective effects of *B. animalis* CGMCC25262 on the skin's physical barrier, the transcription levels of indicators related to barrier functions in HaCaT cells were analyzed using RT-qPCR after co-incubation with various Bifidobacterial samples.

Hyaluronic acid is a naturally occurring biopolymer with multiple functions, including wound repair, cell migration, and signaling. The transcription level of the HAS-2 gene, which encodes hyaluronic acid biosynthesis in HaCaT cells, was significantly upregulated after co-incubation with 0.5–1% CFS of the *B. animalis* CGMCC25262 strain. The highest level of

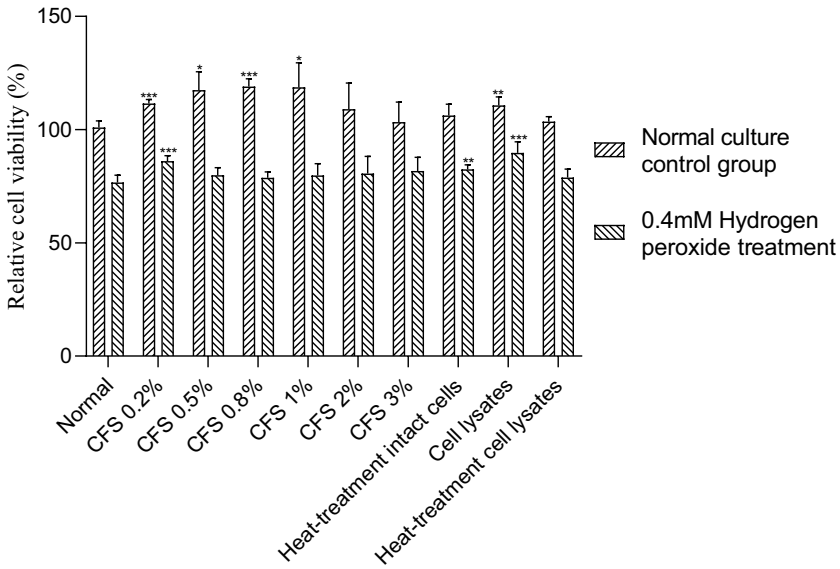

Fig. 1 Detection of proliferative and alleviate oxidative damage effects of different Bifidobacterial samples on HaCaT cells. The normal represents the cells without treating with CFS, bacterial cells or lysates. * $P<0.05$, ** $P<0.01$, *** $P<0.001$

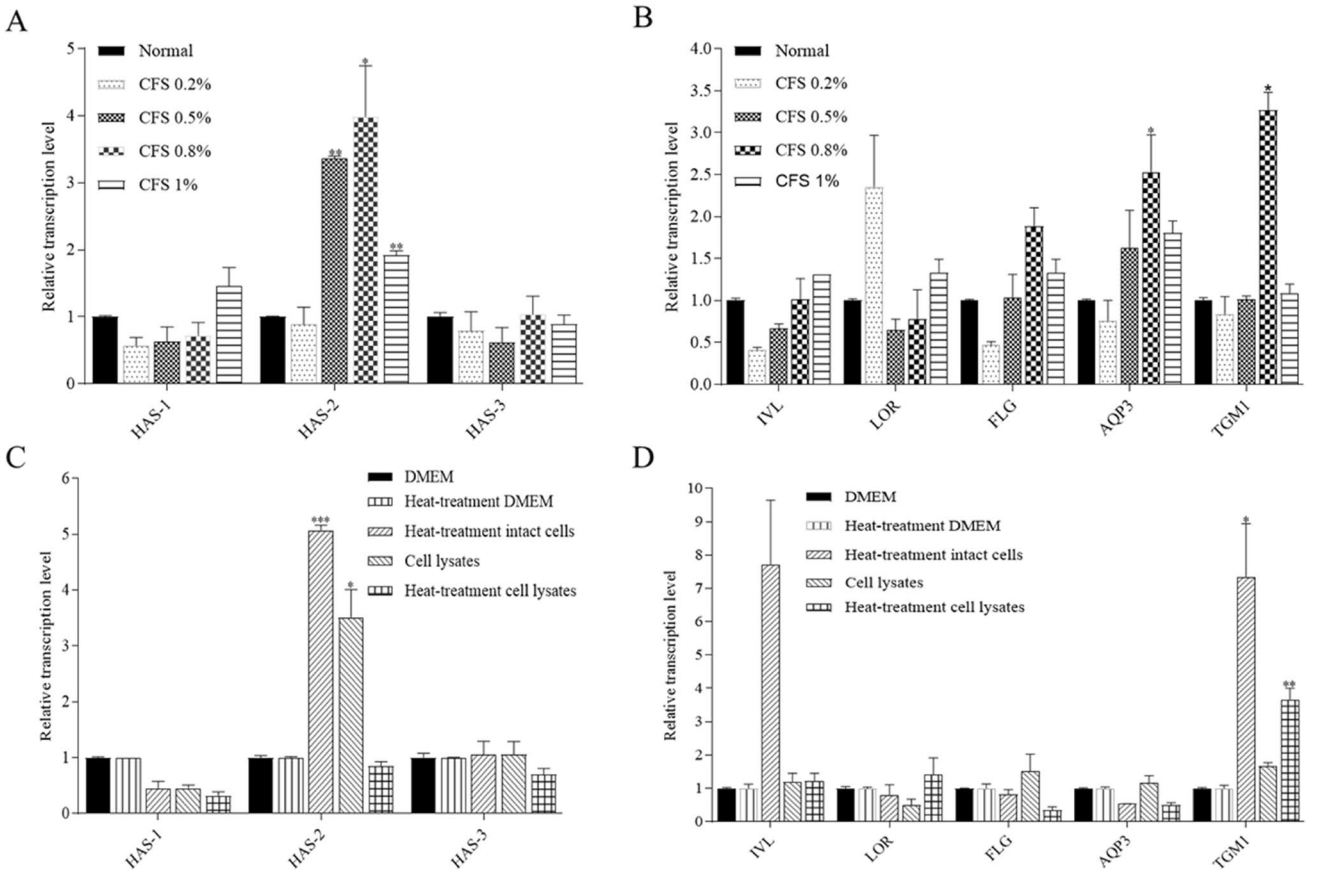

Fig. 2 Transcription levels of genes related to the physical barrier function of the skin were measured in HaCaT cells treated with *B. animalis* samples. **A** Transcription levels of HAS treated with various concentrations of sample 1; **B** Transcription levels of IVL, LOR, FLG, AQP3, and TGM1 treated with sample 1; **C** Transcription levels of HAS treated with sample 2, 3, and 4; **D** Transcription levels of IVL, LOR, FLG, AQP3, and TGM1 treated with sample 2, 3, and 4. * $P<0.05$, ** $P<0.01$, *** $P<0.001$

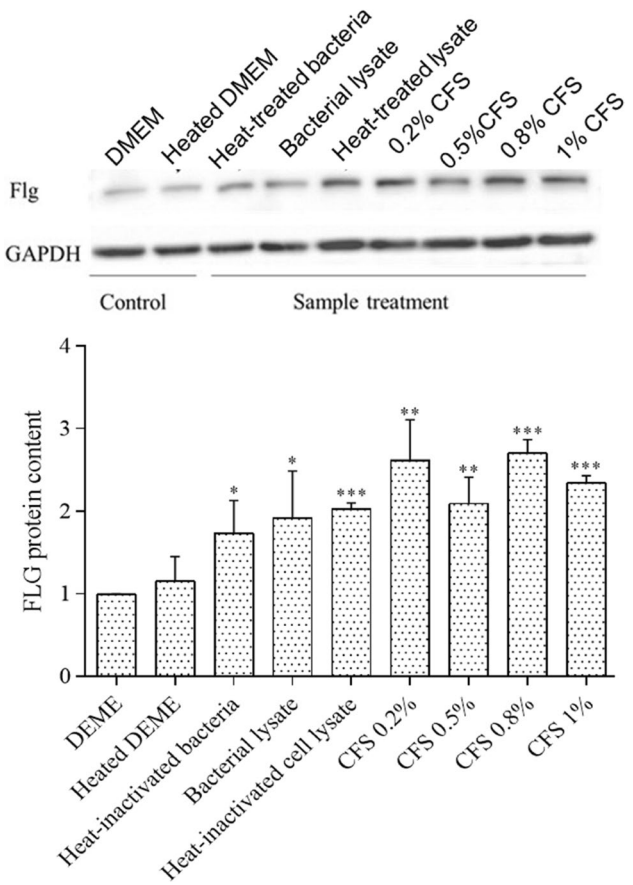

Fig. 3 The protein content of FLG and GAPDH in HaCaT cells when co-incubated with *B. animalis* CGMCC25262 samples. Grayscale values are processed by Image J. * $P<0.05$, ** $P<0.01$, *** $P<0.001$

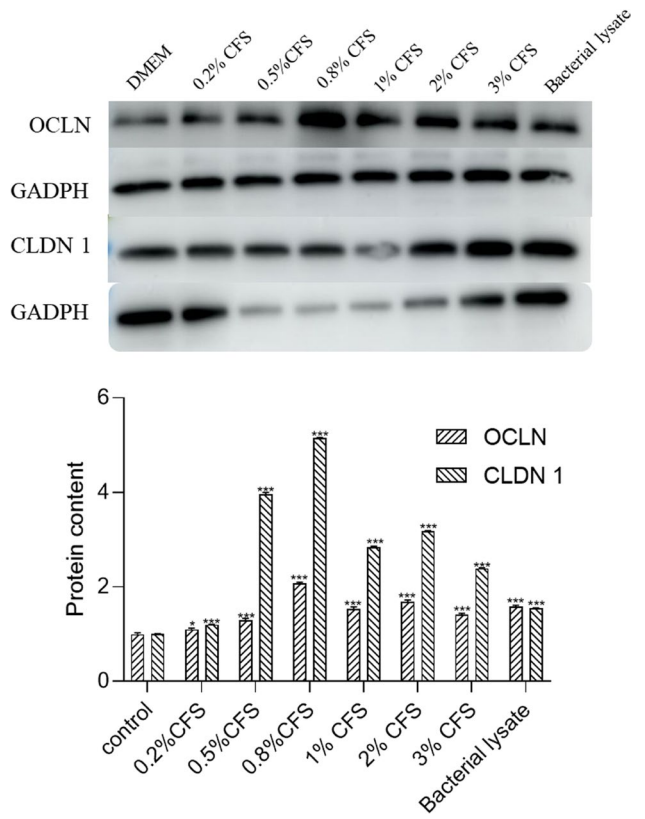

Fig. 4 The protein content of OCLN, CLDN 1, and GAPDH in HaCaT cells when co-incubated with *B. animalis* CGMCC25262 samples. Grayscale values are processed by Image J. * $P<0.05$, ** $P<0.01$, *** $P<0.001$

increase, reaching approximately four times that of HaCaT cells not treated with CFS, was observed when the CFS concentration was at 0.8%. Moreover, an improvement in the transcription levels of genes associated with the skin barrier was observed. This included a twofold increase in LOR levels when treated with 0.2% CFS, a twofold increase in FLG levels, a 2.5-fold increase in aquaporin (AQP3) levels, and a threefold increase in transglutaminase 1 (TGM1) levels when treated with 0.8% CFS. These findings suggest that the CFS of the strain *B. animalis* CGMCC25262 has the potential to stimulate the production of molecules related to skin barrier formation and hydration in human keratinocytes. The optimal concentration for this stimulation falls within the range of 0.5 to 0.8%.

The heat-inactivated bacteria and bacterial lysate also significantly upregulated the transcription level of the HAS-2 gene in HaCaT cells ($P \leq 0.05$). However, the former only upregulated the transcription levels of the IVL gene, which encodes the involucrin protein, and the TGM1 gene, which encodes transglutaminase 1 protein ($P \leq 0.05$). The expression of the IVL gene increased by 7.5-fold, while the expression of the TGM1 gene increased by 7-fold, compared to HaCaT cells alone. Furthermore, both the bacterial lysate and heat-inactivated bacterial lysate showed no obvious promotion effects on the genes related to barrier functions. This suggests that components of bacterial structure of the *B. animalis* CGMCC25262, such as cell wall fragments or cell membrane fragments, also play a protective role in maintaining the skin's physical barrier.

Filaggrin (FLG) is an essential structural protein that plays a crucial role in the development and maintenance of the skin barrier. The expression level of the FLG protein gene in HaCaT cells was significantly increased when co-incubated with different samples of *B. animalis* CGMCC25262. These samples included heat-inactivated bacteria, bacterial lysate, heat-inactivated bacterial lysate, and different concentrations of the CFS compared to untreated HaCaT cells ($P \leq 0.05$). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The CFS showed a stronger promoting ability than that of the heat-inactivated bacteria and cell

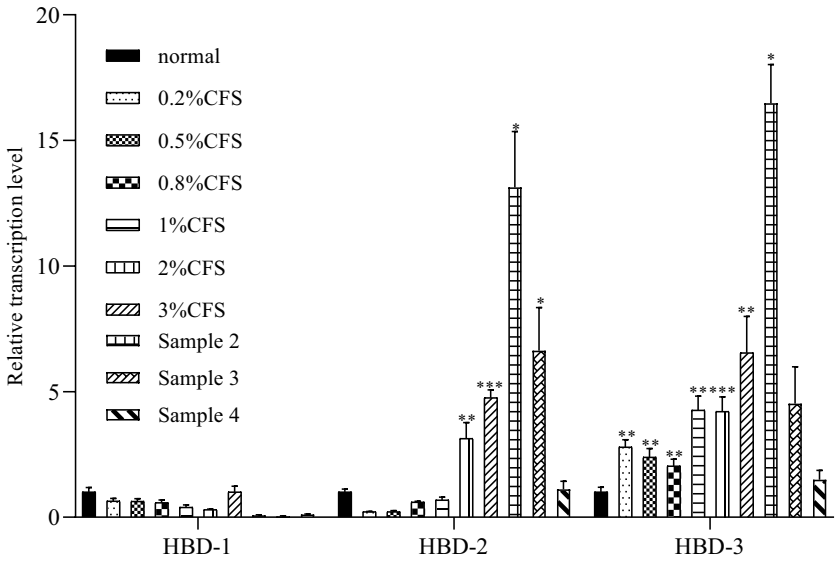

Fig. 5 HBD gene transcription levels in HaCaT cells when co-incubated with various samples. Normal cells were represented without CFS treatment. * $P<0.05$, ** $P<0.01$, *** $P<0.001$

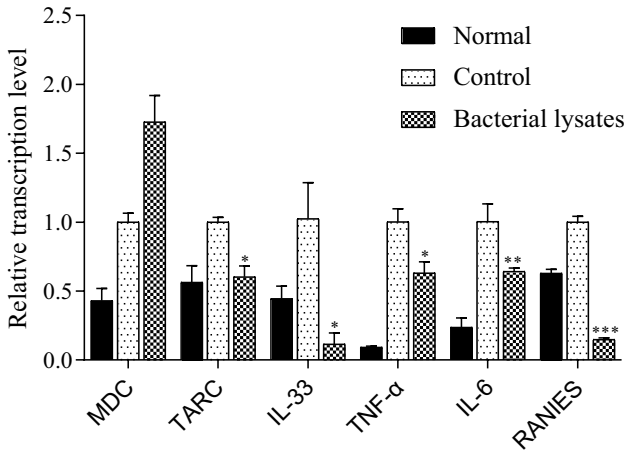

Fig. 6 Relative transcription levels of proinflammatory factors in HaCaT cells when co-incubated with bacterial lysate. * $P<0.05$, ** $P<0.01$, *** $P<0.001$

lysate ($P\leq0.01$). The optimal co-incubated concentration of the CFS fell within the range of 0.2~0.8% ($P\leq0.01$), indicating that *B. animalis* CGMCC25262, particularly its active metabolites, have the ability to enhance the formation of the skin barrier (Fig. 3). This finding is consistent with the observed transcription levels for the FLG gene observed in Fig. 2.

Occludins (OCLN) and Claudins (CLDN) are tight junction proteins, and changes in their expression levels and distribution directly impact the structure and function of tight junction proteins. All of the samples increased the content of OCLN protein, indicating that *B. animalis* CGMCC25262 can enhance the skin barrier (Figs. 3 and 4).

Promoting effects of Bifidobacterial samples on the transcription of genes related to skin chemical barrier

Human β-defensin (HBD) is a small polypeptide primarily synthesized by epithelial cells. It has potential antimicrobial activity against Gram-negative bacteria and *Candida sp.*, but it does not exhibit activity against Gram-positive *Staphylococcus aureus*. Concentrations of 0.2–3% CFS of the strain *B. animalis* CGMCC25262 significantly upregulated the transcription of the HBD-3 gene in HaCaT cells ($P\leq0.01$), as shown in Fig. 5. Furthermore, for HBD-2 gene transcription, a concentration of 2–3% of CFS also resulted in significant upregulation ($P\leq0.01$), as indicated in the same figure.

Similarly, the heat-inactivated bacteria significantly increased the transcription levels of the HBD-2 and HBD-3 genes (Fig. 5). Therefore, it can be concluded that *B. animalis* CGMCC25262 also has a beneficial effect on the chemical barrier of the skin.

Attenuated effects of Bifidobacterial samples on the proinflammatory genes

TNF-α and IFN-γ are proinflammatory cytokines that activate the mitogen-activated protein kinase (MAPK) signaling pathway, resulting in the release of proinflammatory cytokines such as interleukin-6 (IL-6), IL-8, and TNF-α. This cascade can trigger an inflammatory response (Kim et al. 2015; Yano et al. 2015). To investigate the protective effects of *B. animalis* CGMCC25262 on the inflammatory process, we used a combination of TNF-α and IFN-γ was employed to induce an inflammatory-damaged model using HaCaT cells.

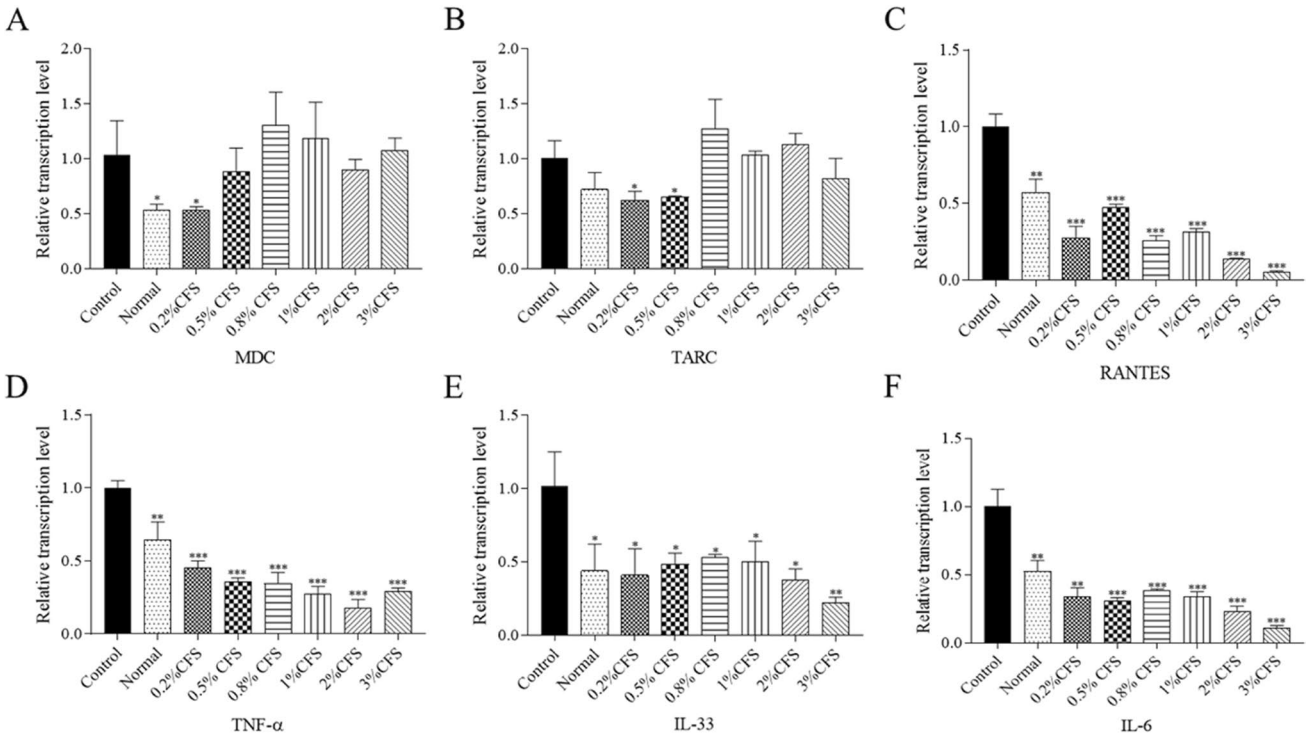

Fig. 7 Relative transcription levels of proinflammatory factors in HaCaT cells when co-incubated with various concentrations of CFS. **A** MDC, **B** TARC, **C** RANTES, **D** TNF-α, **E** IL-33, and **F** IL-6 after CFS treatment with different concentrations. The control was treated with TNF-α and IFN-γ only. Normal meant treated with no samples or inducer. * $P<0.05$, ** $P<0.01$, *** $P<0.001$

As shown in Fig. 6, TNF-α and IFN-γ significantly upregulated the gene transcription levels of major proinflammatory cytokines, including MDC, TARC, IL-33, IL-25, IL-6, TNF-α, and RANTES, which are involved in inflammatory responses in HaCaT cells, compared to untreated HaCaT cells ($P\leq0.05$). This indicates the successful establishment of an inflammatory-damaged model. After co-incubation with the bacterial lysate of *B. animalis* CGMCC25262, the transcription levels of the inflammation-related indicator genes, including RANIES, IL-6, TNF-α, IL-33, and TARC in HaCaT cells, were significantly downregulated ($P\leq 0.05$, $P\leq 0.01$, or $P\leq0.001$), suggesting that the bacterial lysate of *B. animalis* CGMCC25262 can alleviate the inflammatory process in HaCaT cells induced by TNF-α and IFN-γ (Fig. 6).

Similarly, the CFS of *B. animalis* CGMCC25262 also downregulated the gene transcription levels of TARC, RANTES, TNF-α, IL-33, and IL-6 to varying degrees in the inflammatory-damaged model of HaCaT cells. Furthermore, the transcription levels of these genes decreased with the increase in the concentration of the CFS, indicating that the metabolites produced by *B. animalis* CGMCC25262 exerted positive effects in alleviating the inflammatory processes in the skin (Fig. 7).

Alleviation effects of the CFS of Bifidobacterial samples on the inflammatory processes in HaCaT cells

To further validate the alleviating effects of the metabolites produced by *B. animalis* CGMCC25262 on the inflammatory processes in HaCaT cells, we examined the expression of extracellular signal-regulated kinase (ERK) and phosphorylated ERK (p-ERK), which are major components of the MAPK pathway involved in the inflammatory response, using Western blot analysis.

As shown in Fig. 8, the ratio of PERK/ERK increased in the inflammatory-damaged model of HaCaT cells induced by TNF-α and IFN-γ, indicating activation of the ERK pathway. However, after co-incubating the induced-damaged HaCaT cells with 0.5–3% CFS and bacterial lysate of *B. animalis* CGMCC25262, the ratio of PERK/ERK notably decreased ($P\leq0.001$). Furthermore, the PERK/ERK ratio decreased as the increasing concentration of the

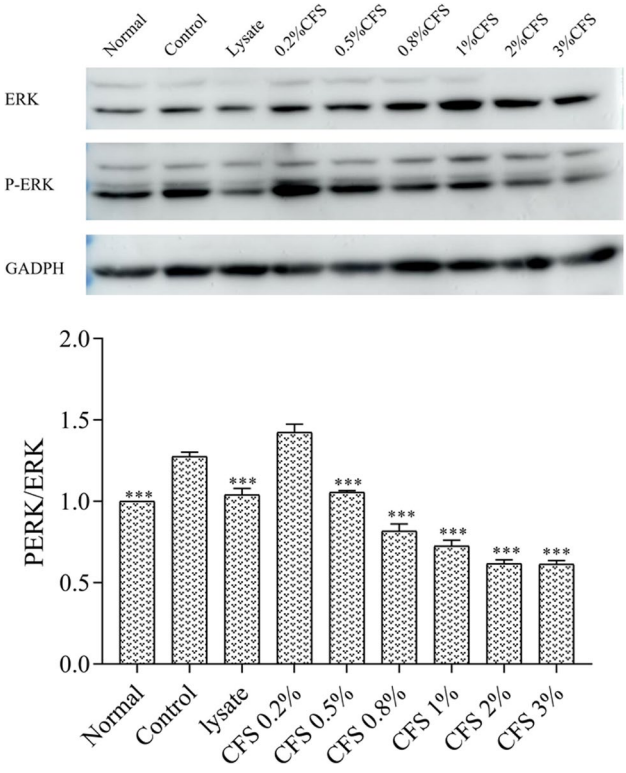

Fig. 8 The protein content of the ERK, PERK, and GAPDH in HaCaT cells after co-incubated with samples of *B. animalis* CGMCC25262. The control group was treated only with TNF-α and IFN-γ, while the normal group was treated with neither samples nor the inducer. Grayscale values are processed by Image J. * $P<0.05$, ** $P<0.01$, *** $P<0.001$

CFS. This result is consistent with the attenuated effects of Bifidobacterial samples on the proinflammatory genes, as shown in Figs. 6 and 7.

Discussion

Postbiotics are non-viable microorganisms or bacterial products produced by probiotics that exhibit health-promoting activities in the host. Effective postbiotics contain inactivated microbial cells or cell components, with or without metabolites (Wegh et al. 2019). Recently, there has been a growing trend of incorporating postbiotics into cosmetic and skincare products. For example, the well-known *Bifida*-ferment lysate, derived from *Bifidobacterium longum*, has demonstrated protective roles against UV-induced skin damage and an anti-aging effect (Hong et al. 2015; Vasiliki and Mihalis 2019; Hong et al. 2022). However, it is worth noting that the probiotic effects can vary depending on the specific strain (Pandey et al. 2015; Suez et al. 2019). In this study, *B. animalis* CGMCC25262, isolated from the intestinal tracts of healthy adults, was experimentally proved to increase the transcription levels of genes associated with the skin barrier function, including HAS-2, AQP3, TGM1, and HBD. Additionally, it downregulates the pro-inflammatory cytokine genes by inhibiting of the ERK phosphorylation signaling pathway in induced-damaged HaCaT cells. This effect is particularly observed when using the bacterial lysate and cell-free supernatant of the *B. animalis* CGMCC25262 strain. Therefore, *B. animalis* CGMCC25262 and its derived components have the potential to be used in the development of cosmetic materials for enhancing skin health.

Postbiotics typically exhibit beneficial health effects through mechanisms similar to those of probiotics, such as preventing infections, accelerating wound healing, and producing anti-inflammatory and immunomodulatory effects (Thorakkattu et al. 2022). Surprisingly, both heat-inactivated bacterial cells and bacterial lysate, as well as 0.2–0.8% CFS of *B. animalis* CGMCC25262, were able to improve HaCaT cell viability. This suggests that the *B. animalis* CGMCC25262 and its metabolites have no harmful effect on the skin. Instead, they appear to provide essential nutrients that support the proliferation of HaCaT cells (Fig. 1). This observation aligns with previous research indicating that *Bifidobacterium* is capable of producing a range of vital compounds, including vitamin B complexes such as thiamine, riboflavin, vitamin B6, vitamin K, folic acid, niacin, and pyridoxine, as well as certain amino acid metabolites like indole-3-lactic acid (ILA) and phenyllactic acid (PLA) during their growth processes (Deguchi et al. 1985; Rossi et al. 2011; Solopova et al. 2020; Sen et al. 2023).

The skin barrier is one of the most important functions in protecting against pathogen invasion and external injury from environmental factors. Some postbiotics could help enhance the skin barrier, and increase the expression of tight junction protein (Rawal and Ali 2023). To assess the probiotic effects of strain CGMCC25262 and its metabolites on the skin barrier, we selected several genes that serve as markers for the physical and chemical barriers to measure their transcriptional levels. Hyaluronic acid is the primary component of skin's extracellular matrix and has a multifaceted role in regulating various biological processes, including skin repair, wound healing, and tissue regeneration (Kakehi et al. 2003; Gomes et al. 2020; Kobayashi et al. 2020; Skandalis et al. 2020). Hyaluronic acid can be synthesized under the catalysis of hyaluronic acid synthetase, including HAS-1, HAS-2, and HAS-3, which are encoded by the genes has-1, has-2, and has-3, respectively. Among these synthetase, HAS-2 is known for its particularly robust catalytic activity (Spicer and Nguyen 1999; Weigel 2002; Siiskonen et al. 2015). As expected, the transcription levels of the genes associated with hyaluronic acid synthesis,

especially Has-2, were significantly upregulated. Similarly, the AQP3, which is responsible for transporting water and uncharged molecules from sebaceous glands to the epidermis to ensure continuous skin moisture, exhibited the same upregulation (Takata et al. 2004; Wang et al. 2006; Hermo and Smith 2011; Abir-Awan et al. 2019). This trend extended to other genes related to the skin's physical barrier, including FLG, loricrin (LOR), involucrin (IVL), OCLN, and TGM1. Consequently, these findings emphasize the probiotic effects of strain *B. animalis* CGMCC25262 and its metabolites in promoting skin barrier repair and enhancing epidermal hydration.

HBD is a type of antibacterial peptide that exhibits a potent inhibitory effect inhibitory effect on bacteria, fungi, and viruses (O'Neil 2003). HBD consists mainly of HBD-1 through HBD-6. HBD-1 is expressed constitutively in the intestinal epithelium and is not affected by external factors, making it an essential defense component. HBD-2 is primarily expressed in the skin and mucous tissues as a response to infections, making it an inducible defensin. HBD-3 is mainly expressed in the oral mucosa, skin, and tonsils. HBD is an important antimicrobial peptide in the chemical barrier of human skin (Park et al. 2018; Ma et al. 2022). The heat-inactivated bacterial cells and bacterial lysate that were tested were able to promote the transcription of the HBD genes to different extents in HaCaT cells. This further validated the protective roles of the strain *B. animalis* CGMCC25262 and its components against pathogen invasion, as well as the maintenance of a healthy skin microecological balance.

Atopic dermatitis (AD) is a prevalent, chronic inflammatory skin disease characterized by symptoms such as pruritus and recurrent skin damage (Sroka-Tomaszewska and Trzeciak 2021). *Bifidobacterium longum* lysate was found to improve the reactive skin (Gueniche et al. 2010). What's more, the use of lysate of probiotic strain *Lactobacillus reuter* DSM 17938 in ex vivo skin models could manage skin inflammation and keep skin healthy (Khmaladze et al. 2019). The complex pathological mechanism behind the etiology of AD involves the destruction of the epidermal barrier due to the deficiency of FLG protein and the infiltration of T cells. This leads to the production of numerous chemokines and cytokines, resulting in skin inflammation (Boguniewicz et al. 2006; Bieber 2022; Gewiss and Augustin 2023). The results showed that all samples of *B. animalis* CGMCC25262 tested, including heat-inactivated bacteria, bacterial lysate, heat-inactivated lysate, and different concentrations of the CFS, were able to promote the gene expression of FLG protein in HaCaT cells compared to untreated HaCaT cells ($P \leq 0.05$). In addition, the gene transcription levels of the inflammation-related indicators RANTES, IL-6, TNF-α, IL-33, and TARC were significantly downregulated. This further confirmed the beneficial effects of *B. animalis* CGMCC25262 samples in alleviating skin inflammation processes.

Conclusion

This study aimed to assess the probiotic properties of *B. animalis* CGMCC25262 on skin health using HaCaT cells as a model. This was accomplished by analyzing the transcription and expression levels of genes associated with the skin's barrier, encompassing both its physical and chemical components. The results from the experiments confirmed that the heat-inactivated bacterial cells, bacterial lysate, and CFS of strain *B. animalis* CGMCC25262 had the ability to improve skin barrier function, specifically by enhancing hydration and moisture retention. Moreover, products derived from the fermentation of strain *B. animalis* CGMCC25262 exhibited the ability to downregulate the transcription of the proinflammatory cytokine gene in keratinocyte HaCaT cells by inhibiting the ERK phosphorylation signaling pathway. The cumulative experimental findings provide strong evidence for the potential of this strain in developing novel biomaterials for skincare products.

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Declarations

Ethic statements The authors declare this research did not include any human subjects and animal experiments.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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